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Influence of Absorption Promoters on Pulmonary Insulin Bioactivity

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ABSTRACT

The purpose of this research was to enhance the bioactivity of insulin by the pulmonary route using a combination of absorption promoters. Aliquots (100 µL) containing 1.0 IU/kg to 7.0 IU/kg doses of porcine insulin solutions with different classes of absorption promoters and combinations of these at 3 concentration levels were instilled intratracheally to the anesthetized rats. Blood concentrations of glucose were measured at specific time points. Out of 3 concentration levels of each of the absorption promoters used, the formulations having the least concentration with the maximum percentage of blood glucose reduction were selected for combining absorption promoters, and their pharmacodynamic parameters related to insulin absorption were determined. The pharmacodynamics of porcine insulin following subcutaneous administration of increasing doses were also determined. The relative pulmonary bioactivity of insulin in phosphate buffer pH 7.4 and citrate buffer pH 3.5 was 11.36% ± 1.27% and 43.20% ± 2.48%, respectively, compared to subcutaneous administration. Relative pulmonary bioactivity of 155.60% ± 5.19% was obtained when oleic acid sodium salt, sodium tauroglycocholate, bestatin, and chymostatin were coadministered in citrate buffer pH 3.5 solution. However, only 61.91% ± 3.21, 67.09% ± 3.23%, 67.24% ± 2.11%, and 69.84% ± 3.02% were obtained, respectively, upon incorporation of these absorption promoters individually. Absorption promoters in combination have significant potential for increasing the pulmonary bioactivity of insulin. These studies support the argument that pulmonary administration of insulin is a viable alternative to subcutaneous administration for diabetic patients.

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INTRODUCTION

Many biologically active peptides have been discovered recently and have attracted attention as new drugs. Because of transport and enzymatic barriers, clinical dosage forms of these peptides have been primarily parenteral forms. Development of sustained release forms of these peptide drugs is also being actively research,[1-3] and the pulmonary route would seem to be a promising alternative for delivering them, because many drugs that are poorly absorbed from other mucosal sites are well absorbed from the lungs.[4] This route of administration offers a number of advantages over the conventional gastrointestinal pathway, including large surface area, extensive vasculature, easily permeable membrane, and low intracellular and extracellular enzymatic activity.[5-8] Recent clinical and preclinical reports reveal that delivery of peptide drugs such as leuprolide acetate and insulin is feasible through the pulmonary route.[9-12] However, the bioavailability of the drugs having relatively high molecular weight is still poor through the pulmonary route compared to the parenteral route. So far little information is available regarding the primary factors determining the rates and extent of pulmonary drug absorption, such as molecular size, pH, charge, ions, solubility, partition coefficient, and proteolytic enzymes. Poor bioavailability through the pulmonary route necessitates administration of several times more than the parenteral dose of insulin,[13] leading to inconsistent blood insulin levels, adverse effects, and insulin wastage. Earlier research on enhancing the insulin absorption[14-17] revealed marginal enhancement in insulin relative to pulmonary bioactivity. These investigations endeavored to achieve pulmonary bioactivity comparable to that found with the parenteral route of peptide drug delivery. Combinations of different classes of absorption promoters were incorporated with the drug solution at pH 3.5 to protect

insulin from degradation in the lungs and make the entire drug available for systemic absorption. Absorption promoters used were sodium caprylate, sorbitan trioleate, oleic acid sodium salt, sodium tauroglycocholate, bacitracin, bestatin, and chymostatin. These absorption promoters enhance the absorption by different mechanisms. Hence, combinations of promoters may enhance the insulin absorption synergistically, and the bioactivity of the drug may increase by many times, meaning that smaller concentrations of these promoters will be required to achieve the same bioactivity. Synergistic effect of absorption promoters may reduce the required dose of insulin by increasing the pulmonary bioactivity. Use of absorption promoters in small concentrations is expected to reduce or eliminate the toxicities associated with them at higher concentrations.

MATERIALS AND METHODS

Insulin porcine (25.5 IU/mg) was donated by Sarabhai Chemicals (Vadodara, India). Citric acid anhydrous (extra pure), sodium tauroglycocholate, sorbitan trioleate, and sodium caprylate were purchased from SD Fine-Chem Ltd (Boisar, India). Oleic acid (cis 9-ocatadecanoic acid) sodium salt, bacitracin, bestatin, chymostatin, and urethane were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany).

Reagents

The following reagents were used:

1. Glucose assay kit (Bayer Diagnostics India Ltd, Vadodara, India).
2. Phosphate buffer pH 7.4 (potassium dihydrogen phosphate 35.44 mmol, sodium hydroxide 20.56 mmol), pH 6.0 (potassium dihydrogen phosphate 50 mmol, sodium hydroxide 5.6 mmol), and pH 5.0 (potassium dihydrogen phosphate 50 mmol, potassium hydroxide 6.0 mmol) were prepared in water for injection.
3. Citrate buffer pH 3.5 (citric acid monohydrate 16.53 mmol, disodium hydrogen phosphate 17.8 mmol) was prepared in water for injection. The ionic strength (μ = 0.056) of all the buffers prepared was the same.

Selection of Animals and Experimental Design

Animal experiments were approved by the Social Justice & Empowerment Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Government of India, New Delhi. Animals were included in the study on the basis of randomization selection technique. The following procedure was adopted before starting the animal studies:

1. Six male albino rats (Swiss strain) weighing between 220 and 230 g were included in each group.
2. Rats were housed in large polypropylene cages at air-conditioned temperatures (22°C-24°C), normal hygiene, and normal diet at a 12-hour light/dark cycle, and water was given ad libitum.
3. Rats in each group were subjected to the experiments on the same day at the same time.
4. Animals were fasted overnight (16 hours) before study. However, water was allowed ad libitum.

Preparation of Formulations

Freshly prepared formulations were used throughout the study.

Subcutaneous Formulations

For subcutaneous administration, 5 formulations of porcine insulin were prepared. The drug was dissolved in citrate buffer pH 3.5, and the solutions were diluted with the same buffer to 100 µL of the final solutions containing 0.50 IU/kg, 0.75 IU/kg, 1.0 IU/kg, 1.25 IU/kg, and 1.50 IU/kg of insulin, respectively.

Intratracheal Formulations

Porcine insulin crystalline powder was dissolved in phosphate buffer pH 7.4, 6.0, and 5.0 and citrate buffer pH 3.5. These solutions were diluted in a buffer of the same pH, and the final volume was adjusted to the required dose of insulin being instilled (**Table 1**: F1-F4). Solutions of sodium caprylate, sorbitan trioleate, oleic acid sodium salt, sodium tauroglycocholate, bacitracin, bestatin, and chymostatin were prepared in citrate buffer pH 3.5. These solutions were added separately or in combination to the insulin in citrate buffer pH 3.5. The solutions were diluted with the buffer of the same pH to 100 µL of the final solutions containing the required dose of insulin and the concentration of absorption promoters (**Table 1**: F5-

Table 1. Formulation Composition and Mean PBGR of Insulin Formulations After Intratracheal Administration*

Formulation	Dose (IU/kg)	Formulation Variables			Mean PBGR
		pH	Penetration Enhancer	Protease Inhibitor	
F1	7.0	7.4	—	—	31.94 ± 2.8
F2	7.0	6.0	—	—	36.49 ± 3.8
F3	6.0	5.0	—	—	44.98 ± 2.8
F4	3.0	3.5	—	—	44.60 ± 2.7
F5	2.5	3.5	Sodium caprylate 0.1%	—	43.05 ± 1.6
F6	2.5	3.5	Sodium caprylate 0.5%	—	45.07 ± 1.0
F7	2.5	3.5	Sodium caprylate 1.0%	—	45.09 ± 1.2
F8	2.0	3.5	Sorbitan trioleate 0.1%	—	29.13 ± 1.2
F9	2.0	3.5	Sorbitan trioleate 0.4%	—	32.28 ± 0.7
F10	2.0	3.5	Sorbitan trioleate 0.7%	—	32.71 ± 1.1
F11	2.5	3.5	Oss 0.1%	—	39.02 ± 1.7
F12	2.5	3.5	Oss 0.3%	—	46.71 ± 1.4
F13	2.5	3.5	Oss 0.5%	—	49.68 ± 0.9
F14	1.5	3.5	Stg 0.1%	—	33.12 ± 1.2
F15	1.5	3.5	Stg 0.3%	—	35.93 ± 0.7
F16	1.5	3.5	Stg 0.5%	—	36.65 ± 0.9
F17	2.0	3.5	—	Bacitracin 0.02%	31.36 ± 1.3
F18	2.0	3.5	—	Bacitracin 0.05%	35.34 ± 1.2
F19	2.0	3.5	—	Bacitracin 0.10%	37.44 ± 1.5
F20	1.5	3.5	—	Bes 0.01%	29.74 ± 0.9
F21	1.5	3.5	—	Bes 0.03%	36.05 ± 1.0
F22	1.5	3.5	—	Bes 0.05%	36.25 ± 1.1
F23	1.5	3.5	—	Chy 0.01%	28.58 ± 1.4
F24	1.5	3.5	—	Chy 0.03%	35.34 ± 1.5
F25	1.5	3.5	—	Chy 0.05%	37.56 ± 0.6
F26	1.0	3.5	Oss 0.1% + stg 0.05%	—	30.05 ± 1.0
F27	1.0	3.5	Oss 0.2% + stg 0.10%	—	31.80 ± 0.7
F28	1.0	3.5	Oss 0.5% + stg 0.30%	—	32.15 ± 1.0
F29	1.0	3.5	—	Bes 0.01% + chy 0.02%	29.82 ± 1.0
F30	1.0	3.5	—	Bes 0.03% + chy 0.05%	32.67 ± 0.8
F31	1.0	3.5	—	Bes 0.02% + chy 0.04%	34.59 ± 1.6
F32	1.0	3.5	Oss 0.2% + stg 0.1%	Bes 0.02%	42.54 ± 1.8
F33	1.0	3.5	Oss 0.2% + stg 0.1%	Chy 0.04%	44.26 ± 1.7
F34	1.0	3.5	Oss 0.2% + stg 0.1%	Bes 0.02% + chy 0.04%	50.28 ± 1.8

*PBGR indicates percent blood glucose reduction; oss, oleic acid sodium salt; stg, sodium tauroglycocholate; bes, bestatin; chy, chymostatin.

F34). The compositions of all the formulations prepared are recorded in **Table 1**.

Control Formulations

Nine control formulations were prepared. Two were phosphate buffer pH 7.4 and citrate buffer pH 3.5, and the other 7 formulations contained the absorption promoters individually in citrate buffer pH 3.5. The concentration of each of the absorption promoters in the control formulations was equal to the maximum concentration of these used in intratracheal formulations.

Subcutaneous Administration

Rats were anesthetized by means of an intraperitoneal injection of urethane (120 mg/100 g). The femoral vein was catheterized using silicone tubing (0.02-mm internal diameter and 0.05-mm outer diameter), and the patency of the catheter was confirmed by slowly flushing the cannula with 200 µL of heparinized saline. Increasing doses of insulin formulations prepared in citrate buffer pH 3.5 (0.50 IU/kg, 0.75 IU/kg, 1.0 IU/kg, 1.25 IU/kg, and 1.50 IU/kg) were administered subcutaneously to each animal group. These doses were chosen because a linear dose-response relationship was seen. The doses above this range resulted in hypoglycemia or fluctuation in peak response time. Blood samples (100 µL) were withdrawn at –60, –30, 0, 30, 60, 90, 120, 150, 180, 210, 240, 270, and 300 minutes through the lateral tail vein. Following each sampling, an equal amount of saline was injected through the catheter tube attached to the femoral vein.

Intratracheal Solution Instillation

After the animals were anesthetized, intratracheal instillation was performed as reported by Enna and Schanker.[18] The rats were placed on a heating blanket thermostatically controlled at 37°C via rectal probe. Before the surgery (–60 minutes of instillation), 1 blood sample was collected from the tail vein. The trachea was exposed by blunt dissection of the sternohyoideus muscle, and a small midline incision was made over the trachea between the fifth and sixth tracheal rings using a 20-gauge needle. The trachea was cannulated with polyethylene (PE) 200 tubing (5-7 cm) with the tip positioned approximately at the tracheal bifurcation. The PE 50 (10-15 cm) tubing connected to a glass Hamilton syringe (Merck Ltd, Mumbai, India) was inserted into the cannula and advanced to the bifurcation of the trachea. The femoral vein was catheterized using silicone tubing (0.02-mm internal diameter and 0.05-mm outer diameter), and the patency of the catheter was confirmed by slowly flushing the cannula with 200 µL of heparinized saline. The absence of reflexes and the breathing rate were visually monitored throughout the experiment. Exactly 100 µL of the formulation solution was instilled through a tube inserted in the trachea by a 500-µL glass syringe. Blood samples (100 µL) were withdrawn at –30, 0, 30, 60, 90, 120, 150, 180, 210, 240, 270, and 300 minutes through the lateral tail vein. After each sampling, an equal amount of saline was injected through the catheter tube inserted in the femoral vein. An insulin syringe was used for the withdrawal of blood samples from the tail vein and transferred to the microcentrifuge tube. At the end of the experiment, the rats were euthanized with an overdose of anesthesia and exsanguinated.

Blood Samples

The blood samples were allowed to clot for 10 minutes and centrifuged at 4000 to 5000 rpm for about 5 minutes in cold centrifuge at 0°C (C-94, Remi Instruments, Mumbai, India). The serum was separated out using a micropipette and transferred to siliconized 1.0-mL Eppendorf tubes. It was refrigerated at 0°C to 4°C until completion of the study for subsequent glucose estimation.

Blood Glucose Determination

The glucose content was measured by the glucose oxidase-peroxidase method.[19] The analysis is based on the enzyme-catalyzed reaction of glucose with molecular oxygen, followed by a second reaction that produces an intense red color. The color intensity is proportional to the amount of oxidized glucose in the sample. This analytical method yielded a serum glucose concentration in the range of 20 to 350 mg/dL with 2% precision. The mean percent blood glucose reduction (PBGR) was calculated from the amount (mg/dL) of blood glucose measured.

Pharmacodynamic Analysis

The $AUC_{0\text{-}300\ min}$ (area under the blood glucose reduction-time curve) of both subcutaneously administered insulin and intratracheally instilled insulin formulations was calculated by the trapezoidal rule.[20]

The maximum percent blood glucose reduction ($PBGR_{max}$) and the time to attain each $PBGR_{max}$

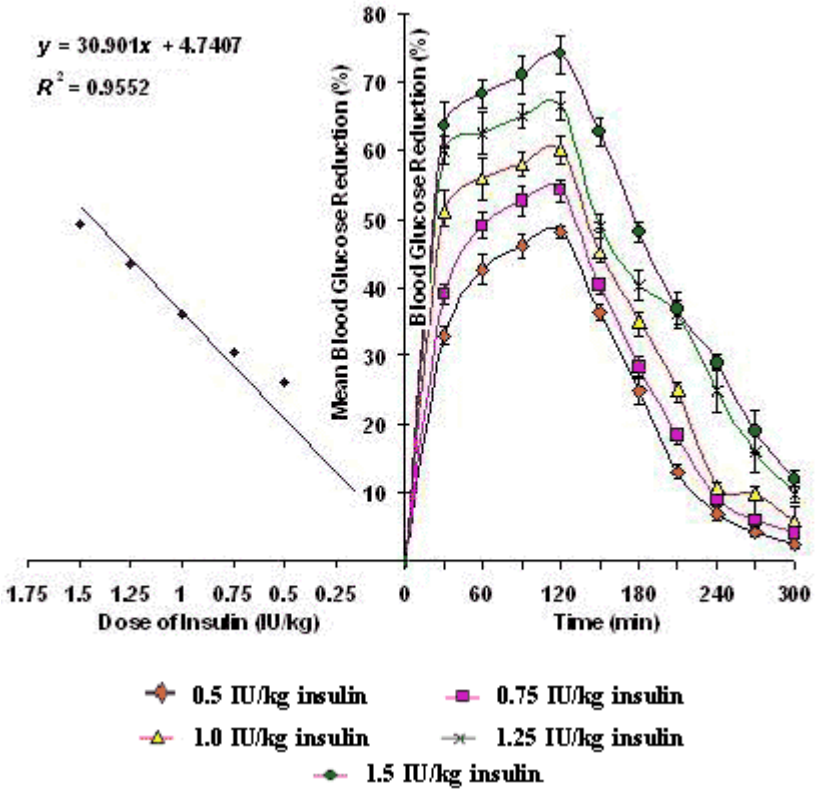

Figure 1. Profiles of blood glucose reduction in rats following subcutaneous administration. Values represent mean ± SE (n = 6).

(T.PBGR$_{max}$) were determined from PBGR-time curves. The percent relative pulmonary bioactivity (F*) with respect to subcutaneously administered insulin was calculated as follows:

$$F^* = \frac{AUC_{0\text{-}300\,min}\ Intratracheal\ Route \times Subcutaneous\ Dose}{AUC_{0\text{-}300\,min}\ Subcutaneous\ Route \times Intratracheal\ Dose} \times 100 \quad (1)$$

Statistical Analysis

Each experiment was conducted on a group of 6 rats, and the mean and SD of the 6 values were recorded. Linear regression analysis was done for finding the correlation among the responses (mean blood glucose reduction) of different doses of subcutaneously administered insulin and shown in **Figure 1**. Tukey's multiple comparisons test[21] was applied individually between the formulations having the same dose of insulin and the same absorption promoters at 3 different concentrations and recorded in **Table 2**.

RESULTS AND DISCUSSION

The subcutaneous formulations of insulin in doses of 0.50 IU/kg, 0.75 IU/kg, 1.0 IU/kg, 1.25 IU/kg, and 1.50 IU/kg were administered subcutaneously, and the PBGR over a period of 30 to 300 minutes is shown in **Figure 1**. The PBGR was found to be continuously increasing up to 120 minutes and then decreasing up to 300 minutes for all the doses of insulin administered. A linear dose-response relationship was observed (r^2 = 0.96). The pharmacodynamic parameters of the subcutaneously administered insulin are recorded in **Table 3**. The T.PBGR$_{max}$ observed was 120 minutes for all the doses of insulin, and the PBGR$_{max}$ was found to increase linearly with respect to dose. The AUC$_{0\text{-}300\ min}$ (7689 ± 369, 8958 ± 403, 10 617 ± 548, 12 754 ± 634, and 14 349 ± 624) was also found to increase in proportion to the dose (0.5-1.5 IU/kg, respectively) administered.

The compositions of various formulations of insulin prepared for intratracheal administration are recorded in **Table 1**. F1 to F4 have 4 different pHs (7.4, 6.0, 5.0, and 3.5) but no other absorption promoters. F5 to F25 have 7 individual absorption promoters (penetration enhancers and protease inhibitors) at 3 concentration levels each.

Table 2. Statistical Data Analysis*

Serial No	Formulations for Comparison	Distance Between Mean PBGR of Testing Formulation	T	Choice of Formulation (Significant)	Selected Formulation*
S1	F5, F6	43.05 – 45.07 = 2.02	> 1.93	F6 or F7	F6
	F5, F7	43.05 – 45.09 = 2.04	> 1.93		
	F6, F7	45.07 – 45.09 = 0.02	< 1.93		
S2	F8, F9	29.13 – 32.28 = 3.15	> 0.63	F9 or F10	F9
	F8, F10	29.13 – 32.71 = 3.58	> 0.63		
	F9, F10	32.28 – 32.71 = 0.43	< 0.63		
S3	F11, F12	39.02 – 46.71 = 7.69	> 2.04	F13	F13
	F11, F13	39.02 – 49.68 = 10.66	> 2.04		
	F12, F13	46.71 – 49.68 = 2.97	> 2.04		
S4	F14, F15	33.12 – 35.93 = 2.81	> 1.42	F15 or F16	F15
	F14, F16	33.12 – 36.65 = 3.53	> 1.42		
	F15, F16	35.93 – 36.65 = 0.72	< 1.42		
S5	F17, F18	31.36 – 35.34 = 3.98	> 1.98	F19	F19
	F17, F19	31.36 – 37.44 = 6.08	> 1.98		
	F18, F19	35.34 – 37.44 = 2.10	> 1.98		
S6	F20, F21	29.74 – 36.05 = 6.31	> 0.60	F21 or F22	F21
	F20, F22	29.74 – 36.25 = 6.51	> 0.60		
	F21, F22	36.05 – 36.25 = 0.20	< 0.60		
S7	F23, F24	28.58 – 35.34 = 6.76	> 1.90	F25	F25
	F23, F25	28.58 – 37.56 = 8.98	> 1.90		
	F24, F25	35.34 – 37.56 = 2.22	> 1.90		
S8	F26, F27	30.05 – 31.80 = 1.75	> 1.36	F27 or F28	F27
	F26, F28	30.05 – 32.15 = 2.10	> 1.36		
	F27, F28	31.80 – 32.15 = 0.35	< 1.36		
S9	F29, F30	29.82 – 32.67 = 2.85	> 1.77	F31	F31
	F29, F31	29.82 – 34.59 = 4.77	> 1.77		
	F30, F31	32.67 – 34.59 = 1.92	> 1.77		

*PBGR indicates percent blood glucose reduction. Selected formulations had smaller concentration of absorption promoters; T, threshold value.

F26 to F31 contain a combination of penetration enhancers or a combination of protease inhibitors at 3 concentration levels. F32 to F34 have a combination of penetration enhancers and protease inhibitors. F5 to F34 have the same pH: 3.5. Doses of insulin incorporated in the formulations were kept between 1.0 IU/kg to 7.0 IU/kg to avoid hypoglycemia and death and to obtain measurable response (ie, minimum 20% PBGR and maximum 80% PBGR) depending on absorption promoters used. Exactly 100 µL of these formulations were intratracheally instilled, and the mean PBGRs are recorded (**Table 1**). Tukey's multiple comparisons test[18] was applied individually between the formulations having the same dose of insulin and the same absorption promoters at 3 different concentrations (**Table 2**). Formulations having a smaller concentration of absorption promoters with significantly high PBGR were selected. In this method, the difference between the mean PBGR of the 3 possible pairwise formulation in the testing group (each group contains 3 formulations) were compared with

Table 3. Pharmacodynamic Parameters After Subcutaneous Administration of Insulin in Rats*

Various Parameters	Dose of Insulin				
	0.5 IU/kg	0.75 IU/kg	1.0 IU/kg	1.25 IU/kg	1.5 IU/kg
Mean PBGR (%)	25.76 ± 1.3	30.06 ± 1.4	35.69 ± 1.9	43.01 ± 2.4	48.43 ± 2.2
$PBGR_{max}$ (%)	48.1 ± 0.9	54.1 ± 1.6	60.1 ± 2.0	66.5 ± 2.1	74.0 ± 2.8
$T.PBGR_{max}$ (min)	120	120	120	120	120
$AUC_{0\text{-}300\ min}$ (% min)	7689 ± 369	8958 ± 403	10 617 ± 548	12 754 ± 634	14 349 ± 624

*PBGR indicates percent blood glucose reduction; $PBGR_{max}$, maximum percent blood glucose reduction; $T.PBGR_{max}$, time to attain each $PBGR_{max}$; $AUC_{0\text{-}300\ min}$, area under the blood glucose reduction–time curve of subcutaneously administered insulin over 0 to 300 minutes.

their threshold value T (calculated from the SD of the respective formulations). From each three formulations compared, either one or two were chosen which are significant. Further, from the significant formulations, the formulation with the smaller concentration of absorption promoter was selected. The pharmacodynamic parameters of these selected formulations along with other formulations (F1-F4 and F32-F34) are recorded in **Table 4** (S1-S16).

Figure 2 shows the influence of pH on PBGR after intratracheal administration of insulin formulations (F1-F4), where the calculated PBGR values corresponding to a dose of 3.0 IU/kg insulin were plotted. The maximum reduction at all sampling points was observed in the formulation with pH 3.5 even though it had the lowest dose of insulin (3.0 IU/kg). The pulmonary bioactivity of the formulation (F4) having pH 3.5 was 43.20% ± 2.48% and was found to be decrease to 21.63% ± 1.28%, 14.57% ± 1.51%, and 11.36% ± 1.27%, respectively, with increase in pH (F1-F3 in **Table 4**). In neutral pH, the insulin molecule is typically associated to form hexameric units, a process called fibrillation. When the pH decreases, fibrillation decreases and the insulin molecules exist in only monomeric form.[22,23] Insulin is more stable at acidic pH, and the monomeric form absorbs rapidly in the alveolar region of the lungs.[24,25] Lowering the pH also causes paracellular permeability, possibly by displacing Ca^{2+} from the tight junction. Hence, acidic pH was observed to favor higher penetration of insulin through the alveolar membrane and the pH of subsequent formulations (F5-F34) was 3.5.

Figure 3 shows the influence of different absorption promoters on PBGR after intratracheal administration of selected insulin formulations (**Table 4**: S5-S11), where the calculated PBGR values corresponding to a dose of 1.0 IU/kg insulin were plotted. In **Figure 4** the influence of combinations of absorption promoters on PBGR of the selected insulin formulations (**Table 4**: S12-S16) is shown. The formulations with oleic acid sodium salt (F13) and sodium tauroglycocholate (F15) as penetration enhancer showed more effect on PBGR in comparison to sodium caprylate (F6) and sorbitan trioleate (F9). The protease inhibitors bestatin (F21) and chymostatin (F25) showed more effect on PBGR compared to bacitracin (F19). Hence, oleic acid sodium salt, sodium tauroglycocholate, bestatin, and chymostatin were chosen for the combination studies of absorption promoters at pH 3.5.

The concentration of oleic acid sodium salt in F13 was 0.5% with bioactivity of 61.91% ± 3.21% and of sodium tauroglycocholate was 0.3% in F15 with bioactivity of 67.09% ± 3.23% (**Table 4**). Both of these enhancers were used in F27, where the concentration of oleic acid sodium salt and sodium tauroglycocholate was 0.2% and 0.1%, respectively, and the bioactivity obtained was 79.25 ± 4.31%. Even though the concentration of individual penetration enhancers was reduced by more than 50%, bioactivity increased significantly. This synergistic increase may be due to more than 1 mechanism involved in enhancing drug absorption. The bile salts and fatty acid salts act by reverse micellar binding with subsequent formation of hydrophilic channels in the tight junction.[26,27] The change in paracellular path and formation of hydrophilic channels result in an increase in transepithelial flow. Bile salts also enhance the absorption[28] by binding Ca^{2+} to increase paracellular permeability[29] and by inhibiting proteases like aminopeptidases.[30] The sodium tauroglycocholate used as enhancer was less irritating and its absorption profile more acceptable.[31]

Similarly, when the protease inhibitors bestatin and chymostatin were coadministered with insulin (F31), the bioactivity obtained was 95.51% ± 4.77% compared to

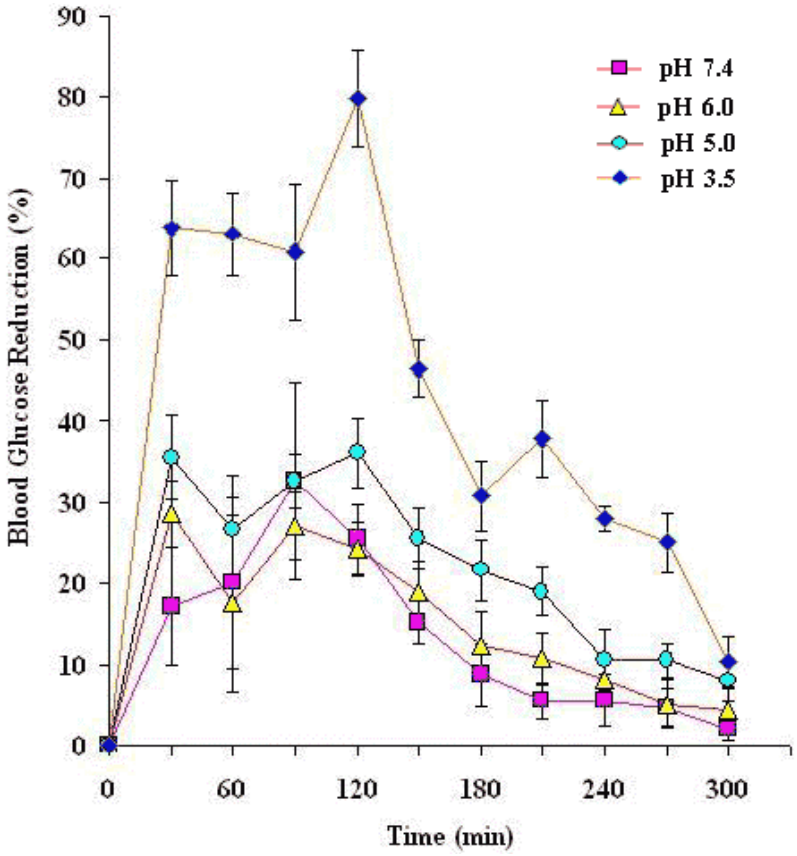

Figure 2. Influence of pH on blood glucose reduction-time profile of intratracheally administered insulin calculated for the dose of 3.0 IU/kg. Values represent mean ± SE (n = 6).

incorporation of bestatin (F21: 67.24% ± 2.11%) and chymostatin (F25: 69.84% ± 3.02%) individually. The surface of a wide variety of mammalian cell types, including lung, are rich in a group of proteolytic enzymes that includes aminopeptidases, carboxypeptidases, dipeptidyl-peptidases, peptidyl-dipeptidases, dipeptidases, and omegapeptidases.[32] These enzymes are responsible for the hydrolysis of peptide drugs administered to the lungs. Bestatin is an aminopeptidase inhibitor, and chymostatin is a serine protease inhibitor. When a combination of these protease inhibitors was used, they inhibited a wide variety of enzymes involved in the degradation of insulin. Hence, the drug degradation was highly protected from the proteolytic enzymes and an increase in bioactivity was observed. The protease and peptidase inhibitors act through inhibition of proteolytic enzymes, and some of them are already approved as therapeutic agents.[31]

When the penetration enhancers oleic acid sodium salt (0.2%) and sodium tauroglycocholate (0.10%) and the protease inhibitors bestatin (0.02%) and chymostatin (0.04%) were combined in an insulin formulation (F34) in citrate buffer pH 3.5, a significant increase in bioactivity of 155.60% ± 5.19% was observed (**Table 4**). Higher bioactivity of formulations with combinations of absorption promoters may be due to degradation of insulin in the subcutaneous tissue,[33,34] partial inhibition of the proteolytic enzymes of lungs, and dilation of the tight junction of the alveolar membrane.

The dose of insulin used in the formulations was found to affect the consistency of PBGR profile significantly. The doses of F26 to F34 are 1.0 IU/kg and the $T.PBGR_{max}$ was found to be 120 minutes for all the formulations (**Table 3**). In **Figure 4**, the PBGR of these formulations increases up to 120 minutes and decreases from 120 to 300 minutes and resembles the subcutaneous PBGR-time profile (**Figure 1**). In formulations with high doses of insulin (1.5 IU/g or more; F1-F25), the $T.PBGR_{max}$ was found to be inconsistent, and interanimal variation of $T.PBGR_{max}$ was 20% to 57%. The PBGR values of these formulations fluctuate significantly, as seen in **Figures 2** and **3**. It is difficult to assign

Table 4. Pharmacodynamic Parameters After Intratracheal Administration of Different Formulations of Insulin*

Serial No	Formulation	$AUC_{0\text{-}300\ min}$ (% min)	$PBGR_{max}$ (%)	$T.PBGR_{max}$ (min)	F*
S1	F1	9513 ± 1031	76.0 ± 0.8	90 ± 30	11.36 ± 1.27
S2	F2	10 825 ± 1109	66.6 ± 4.4	60 ± 30	14.57 ± 1.51
S3	F3	13 261 ± 804	72.0 ± 2.1	70 ± 45	21.63 ± 1.28
S4	F4	13 224 ± 823	79.9 ± 5.1	80 ± 45	43.20 ± 2.48
S5	F6	13 196 ± 672	76.7 ± 3.7	105 ± 21	51.73 ± 2.70
S6	F9	9583 ± 571	54.3 ± 1.5	105 ± 21	53.49 ± 3.18
S7	F13	14 806 ± 765	78.6 ± 4.1	90 ± 30	61.91 ± 3.21
S8	F15	10 685 ± 509	60.1 ± 2.1	90 ± 30	67.09 ± 3.23
S9	F19	10 753 ± 825	78.6 ± 5.3	75 ± 21	50.64 ± 3.90
S10	F21	10 708 ± 613	60.9 ± 3.1	105 ± 21	67.24 ± 2.11
S11	F25	11 122 ± 478	62.3 ± 0.9	105 ± 21	69.84 ± 3.02
S12	F27	9466 ± 678	56.3 ± 3.3	120 ± 00	79.25 ± 4.31
S13	F31	10 140 ± 489	57.9 ± 3.1	120 ± 00	95.51 ± 4.77
S14	F32	12 615 ± 496	64.8 ± 1.8	120 ± 00	123.63 ± 5.13
S15	F33	13 126 ± 495	66.7 ± 2.0	120 ± 00	128.64 ± 4.75
S16	F34	14 889 ± 510	73.9 ± 1.5	120 ± 00	155.60 ± 5.19

$AUC_{0\text{-}300\ min}$ indicates area under the blood glucose reduction–time curve of intratracheally administered insulin over 300 minutes; $PBGR_{max}$, maximum percent blood glucose reduction; $T.PBGR_{max}$, time to attain each $PBGR_{max}$; F, percent relative pulmonary bioactivity.

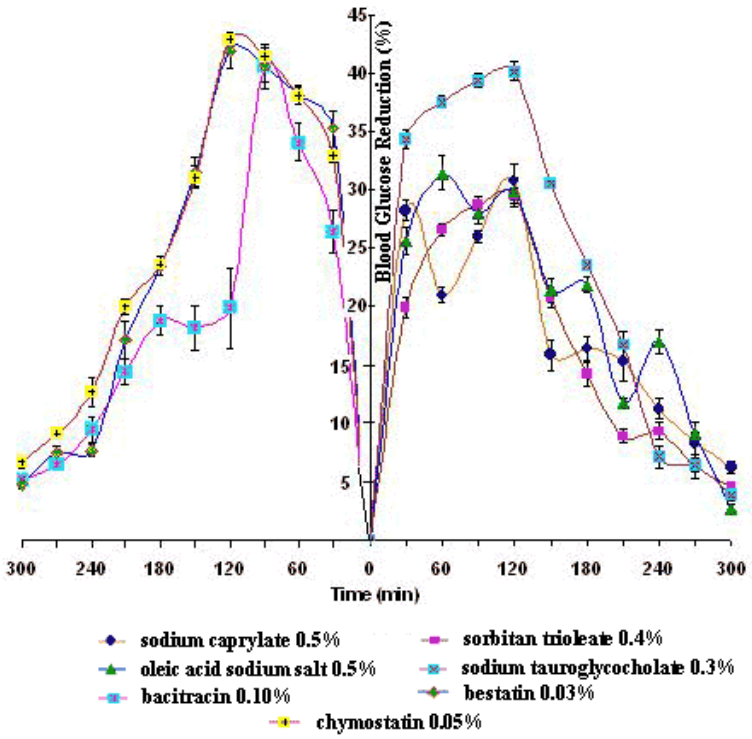

Figure 3. Effects of penetration enhancers and protease inhibitors on blood glucose reduction-time profile of intratracheally administered insulin calculated for the dose of 1.0 IU/kg. Values represent mean ± SE (n = 6).

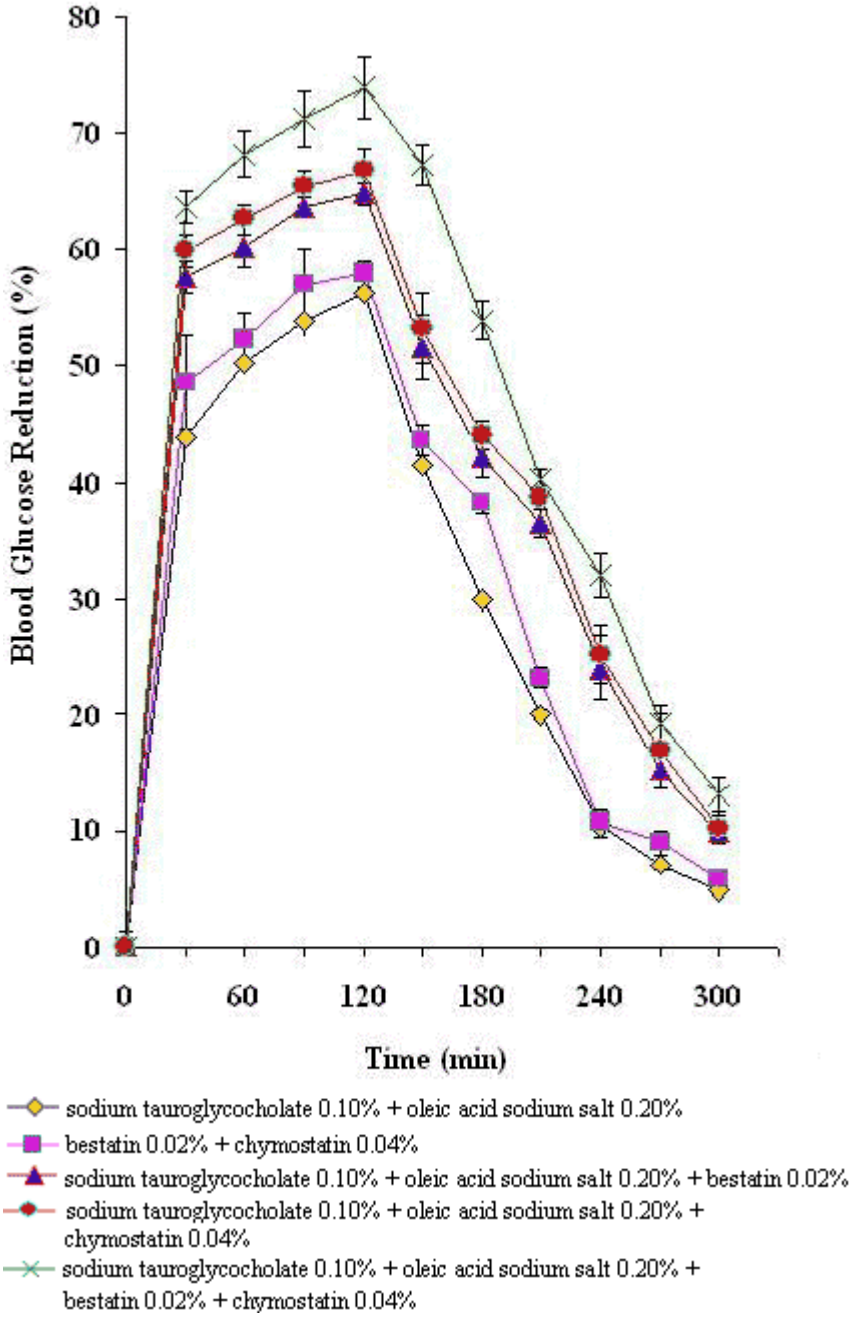

Figure 4. Profiles of blood glucose reduction following intratracheal administration of 1.0 IU/kg insulin with combinations of absorption promoters. Values represent mean ± SE (n = 6).

a specific reason for these fluctuations in glucose levels noticed with higher insulin doses (1.5 IU/kg and above). However, these fluctuations may be due to the body's preventive mechanism in which when the blood glucose level drops below a certain point, the glucose supply from the body's glycogens is triggered and glucose levels again go down because insulin is present in the required concentration. The PBGR-time profile was more consistent for the formulations having a combination of absorption promoters. This increased consistency may be due to the reduction in insulin dose and combination of absorption promoters present in substantially lower concentration, contributing significantly to the safety, efficacy, and reproducibility of the formulations' pharmacological response.

In a group of animals, a surgical operation similar to intratracheal instillation of formulations was performed but no formulations were administered. The glucose levels were monitored over a period of 5 hours. The variation in blood glucose level was found to be within ± 5%. It may be due to the physiological variation observed in basal glucose level. Of the 9 control solutions studied, 7 contained the absorption promoters used in this study, and the remaining 2 contained phosphate buffer pH 7.4 and citrate buffer pH 3.5. The variation in PBGR was found to be within ± 5%.

The data from these studies reveal that the absorption promoters significantly affect the bioactivity of intratracheally administered insulin. This effect depends on the dose of insulin and the concentration and type of the absorption promoters used. The formulation (F34) developed with a combination of protease inhibitors and penetration enhancers in citrate buffer pH 3.5 showed the highest pulmonary bioactivity: 155.60% ± 5.19%. The selected combination of absorption promoters provided a synergistic effect. Nonionic surfactants and fatty acid salts have been reported to have low toxicity,[35] and reduction in the concentration of these absorption promoters demonstrated in these studies makes them even more interesting for human evaluation. Higher bioactivity may decrease the dose of intratracheally administered insulin, help prevent systemic side effects, and reduce the cost of therapy. Findings of these investigations may help in development of dry powder inhalers (DPI) or metered dose inhalers (MDI) of insulin for efficient pulmonary delivery.

However, issues to be investigated prior to development include assessment of stability and reproducibility upon-dosing by pulmonary devices (DPI, MDI, or any other means) and assessment of safety following chronic pulmonary administration.

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